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(57) Abstract

The present invention relates generally to transgenic grass and to a method of producing same. More particularly, the present invention is directed to transgenic grass of the group *Monocotyledoneae*. The transgenic grass of the present invention exhibits the potential to express a range of beneficial traits such as reduced allergenicity, enhanced nutritional content and increased disease resistance.

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TRANSGENIC GRASSES

The present invention relates generally to transgenic grass and to a method of producing same. More particularly, the present invention is directed to transgenic grass of the group *Monocotyledoneae*. The transgenic grass of the present invention exhibits the potential to express a range of beneficial traits such as reduced allergenicity, enhanced nutritional content and increased disease resistance.

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

The rapidly increasing sophistication of recombinant DNA techniques is greatly facilitating the research and development of a range of biotechnology-related industries. The agricultural industry has been a particular beneficiary of recombinant technology especially in crop improvement and in floriculture.

Grasses are one of the most important agricultural plants in the world.

Plants of the group *Monocotyledoneae* (plants of this group are hereinafter referred to as a "Monocots") are particularly important and include members of the family *Poaceae* (Gramineae) such as food crops (for example maize, wheat, rice) and forage grasses (for example, *Lolium, Festuca* and *Dactylis*). They are, however, very difficult to manipulate *in vitro* and to genetically transform (Potrykus, 1990; 1991). Direct DNA injection into young floral tillers and systems involving a mixture of pollen and exogenous DNA to

obtain genetic transformation (Ohta, 1986) have not proven generally applicable in monocots.

Transfer of foreign genes into plants using *Agrobacterium tumefaciens* is routine for many dicotyledonous (dicot) plants. However, this procedure is not routinely applicable in monocots (De Cleene and DeLey, 1976). One reason for this may be the way monocot plants respond to wounding. In dicots, the cells adjacent to wounding de-differentiate and, after incorporating the plasmid DNA, are inducable to form regenerable callus tissue. However, in monocots, wounding leads to the death of the wound-adjacent cells making infection very difficult (Potrykus, 1991). There is a need, therefore, to develop suitable protocols for transforming cells of monocot plants with a capacity to regenerate into whole plants.

Direct gene transfer into protoplasts using electroporation or polyethylene glycol has been successful in regeneration of cereals such as rice (Rhodes et al, 1988) and maize (Shimamoto et al, 1989). However, this method has only been successful in a few cultivars of these crops and regeneration of cereal plants from protoplasts appears to be strongly genotype and culture dependent. In case of rye grass *(Lolium multiforum)*, confirmed stably transformed callus clones from protoplasts have been obtained using neomycin phosphotransferase (npt II) chimeric gene constructs. However, plants could not be regenerated from these calli (Potrykus *et al*, 1985).

Regeneration of plants from single cells or protoplasts is essential in the genetic manipulation of plants using direct gene transfer technology.

Regeneration of plants from non-transformed callus colonies derived from protoplasts have been reported for the monocot forage grasses, *Lolium* and *Festuca arundinacea* (Dalton, 1988a;b), however, such systems are generally not commercially applicable. Genotypic variation (Dale, 1980; Lorz et al, 1985) is also a particular problem in outbreeding species such as commonly occurring ryegrasses because each seed-derived embryo possesses a unique genotype. It has been shown that standard culture conditions to induce embryogenesis are not optimal for every genotype (Dale, 1980). In addition,

protoplast and cell suspension colonies of *Lolium* lines formed virtually only albino shoots (Dalton, 1988a;b) highlighting the difficulty of obtaining functional regenerated plants.

Thus, the generation of plants from cells represents a significant further problem in the production of genetically transformed plants with desirable characteristics. In addition, selection of transformants is often difficult since non-transformed cells are frequently resistant to the selection agents now commonly employed.

Use of microprojectile bombardment allows recovery of transgenic plants without the constraints of protoplast culture and *Agrobacterium* host-specificity. Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells. The concept was first described by Klein *et al* (1987) and has become a successful DNA delivery method in a number of plants.

However, despite its apparent success, the technique has not until the advent of the present invention, been routinely successful in a broad range of monocot grasses.

As stated above, monocot grasses are important agriculturally both at the commercial and environmental levels. Grass pollen, on the other hand, is a significant contributor of respiratory disorders such as hayfever and other allergies with concomitant major downstream health costs including lost production time.

There is a need, therefore, to develop genetic transformation and plant regeneration systems for monocots and in particular monocot grasses so that these plants can be generally manipulated to introduce beneficial traits or to reduce unwanted characteristics. In accordance with the present invention, an effective transformation and regeneration system has been developed for monocot grasses by the inventor permitting the production of transgenic forms of these plants.

Accordingly, one aspect of the present invention relates to a transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species.

More particularly, the present invention is directed to a transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species wherein said transgenic monocot grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic.

Even more particularly, the present invention provides a transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species wherein said transgenic monocot grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic and wherein the callus is subjected to genetic transformation and regeneration on a solid support.

In accordance with these aspects of the present invention and further aspects described and disclosed hereinafter, preferred altered characteristics include but are not limited to altered biochemical or physiological properties, altered nutritional properties of the grass or seeds thereof and altered allergenicity of the grass or its seeds or pollen. For example, characteristics contemplated herein include, but are not limited to, the following:

(a) resistance to plant pathogens;

(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;

(c) enhanced sulphur content;

(d) enhanced expression of proteinase inhibitors;

(e) expression of pigment genes;

(f) expression of regulatory genes to control indigenous genes; and

(g) reduction in levels of allergenic proteins in, for example, pollen.

An "altered characteristic" is readily determined by comparing a transgenic monocot grass with a non-transgenic grass of the same species. The comparison may be at the biochemical, physiological or visual level.

A monocot grass of the present invention includes any grass falling within the major subfamilies, namely the *Bambosoideae* which includes bamboo, *Arundinoideae* which includes pampas grass and reeds, *Pooideae* which includes oats, barley, rye, wheat, corn and the forage grasses fescue (*Festuca*) and ryegrass (*Lolium*), *Chloridoideae* which includes millet, t'ef, Mitchell grass, Rhodes grass, Bermuda grass and Kallar grass and *Panicoideae* which includes sorghum, maize, surinam grass, Pangola grass, Buffel grass, Bahia grass, sugar cane and Vetiver grass. Although the present invention is described and exemplified herein in relation to ryegrass, the techniques of transformation and regeneration are broadly applicable to any of the grasses as contemplated above. In a preferred embodiment, the present invention does not extend to barley, wheat or corn.

In a particularly preferred embodiment, the present invention contemplates a transgenic plant having altered characteristics by the introduction of non-indigenous DNA. A "non-indigenous" DNA is one not normally resident in the plant before transformation or is not normally present in more than one copy. A further copy of an indigenous gene or genetic sequence may be introduced for the purposes of co-suppression. A non-indigenous gene is also referred to as a foreign gene and includes genetic sequences classically corresponding to a "gene" or a part thereof or may be a nucleic acid molecule which in some way effects a change in the transgenic plant. Examples of foreign genes include:

(a) a resistance gene against plant viruses, bacteria, fungi, nematodes and other pathogens;

(b) a gene to improve nutritional value of plants such as sunflower high sulphur gene SF8;

(c) a bloat resistance gene;

(d) an antibody gene (also referred to as a "plantabody");

(e) a cereal thionin and ribosome inactivating protein gene;

(f) an insect resistance gene including BT toxin gene and proteinase inhibitor gene from *Nicotiana alata;*

(g) a selectable marker gene such as those conferring resistance to kanamycin, phosphinothricin, spectinomycin and hygromycin;

(h) a reporter gene such as GUS, CAT and pigment genes;

(i) a gene encoding a regulatory protein which modulates expression of a gene in plant cells;

(j) genetic sequences for co-suppression or having antisense properties; and

(k) genetic sequences for reducing expression or translation of allergenic proteins such as *Lol* proteins including but not limited to *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI.

The genetic techniques of the present invention also contemplate modified plants having indigenous genetic sequences deleted or otherwise mutated. For convenience, such a plant is encompassed hereby by the term "transgenic" in relation to monocot grasses. Such a transgenic monocot grass includes grasses having reduced or modified allergenic proteins such as *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI.

Another aspect of the present invention contemplates a method for producing a transgenic monocot grass, said method comprising deriving callus from a monocot grass and subjecting same to microparticle bombardment and/or *Agrobacterium*-mediated genetic transfer and then placing said callus under conditions sufficient to permit regeneration of said callus into plantlets.

More particularly, the present invention is directed to a method for producing a transgenic monocot grass having at least one altered characteristic compared to a non-

transgenic monocot grass of the same species, said method comprising obtaining callus from a monocot grass on a solid support, introducing into cells of said callus on said solid support genetic material effecting said altered characteristic *via* microparticle bombardment and/or *Agrobacterium* mediated transfer, subjecting said callus to selection conditions and then subjecting callus to regeneration conditions to form plantlets.

In a particularly preferred embodiment, the selection conditions comprises antibiotic or chemical resistance where a gene encoding resistance is on the introduced genetic sequence or vector carrying same. Most preferably, the selection conditions comprise resistance to at least two antibiotics or at least one antibiotic and at least one chemical agent.

Preferably the callus is prepared from mature and/or developing embryos obtained from seeds. More preferably the callus is grown on a callus induction medium which is based on that of Murashige and Skoog (1992) [MS medium] but contains 2,4-dichlorophenoxy acetic acid (2,4D) in a range from about 1 to about 10 mg/L, preferably about 5mg/L, L-asparagine (at about 150mg/L) or equivalent amino acid or chemical entity and preferably thiamine HCl (at about 0.5 mg/L) or equivalent. The medium may also contain MS medium minerals and/or, in a particularly preferred embodiment, potassium iodide or equivalent or functional analogue at 0.83 mg/L.

Preferably, the cells selected for transformation are the healthy cells on the callus and this is facilitated by excision of the embryo and adjacent cells. More preferably, the healthy cells are selected for transformation at an age of between 1 to 10 weeks, more preferably 3 to 6 weeks and most preferably at about 4 weeks old. A particular advantage of the present invention is that the genetic transfer and regeneration is conducted with the callus on solid medium such as agar and/or filter paper. A suspension culture is not first prepared as commonly occurs in the prior art.

The callus is generally from cells which have been either directly or indirectly obtained from the monocot grass tissue. This may be from embryo tissue, root, shoot including leaf tissue and inflorescence tissues. Preferably the callus is derived from mature and/or developing embryos or seeds.

Preferred monocot grasses include forage grass such as but not limited to ryegrass *Lolium* (ryegrass), *Agaropyron, Phalaris, Poa, Festuca, Dactylis, Aleopecuris* and *Phleum.*

Genetic material contemplated herein include vectors suitable for introduction into cells by microparticle bombardment and/or *Agrobacterium* mediated transformation. Although *Agrobacterium* mediated transformation on its own has yet to be widely used in monocots, in accordance with the present invention, the combination of microparticle bombardment and *Agrobacterium* mediated transformation is contemplated to be particularly useful as well as microparticle bombardment on its own. Suitable vectors will be well known by those skilled in the art and are described, for example in Croy R.R.D. (Ed) Plant Molecular Biology, Bios Scientific Publications, Blackwell Scientific Publication (1993).

A particularly useful vector for microparticle bombardment is pBIN19 or related vector or a pUC-based vector such as pUC18 or pUC19 or equivalent or functional analogue.

Preferably, the genetic material effects a reduction in levels of ryegrass pollen allergens such as *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI (see, for example, International Patent Application No. PCT/AU89/00123).

Where the selectable marker encoded by the construct confers resistance to an antibiotic it preferably encodes resistance to a class of antibiotics. This feature allows selection with two or more antibiotics which is a particularly preferred feature of the present invention. This provides a way of overcoming the problem of untransformed plant cells being able to grow on the selected medium. For example, the construct may encode the

gene for neomycin phosphotransferase, npt II,or other antibiotic resistance genes such as hygromycin resistance, aph 3'II or aph IV amongst others. Preferably, the gene is coupled to an appropriate promoter such as pEmu, Ubi I, Act I or the CaMV 35S promoter.

Where neomycin phosphotransferase is encoded by the construct, then generally kanamycin and geneticin are used in the selection medium. Preferably, these antibiotics are present in the amount 15 to 50 mg/L kanamycin and 15 to 50 mg/L geneticin.

A further aspect of the present invention contemplates a method of regenerating monocot grass plants from transformed plant cells, said method comprising contacting cells on a solid support upon which said cells are subjected to transformation means on a medium and under appropriate conditions and time wherein said medium contains shooting and rooting hormones and then culturing the resultant shoots on a medium in the absence of biologically active hormones.

The medium which has an absence of hormones may be any suitable medium and includes soil or potting mix.

A rooting hormone is a class of compounds known as auxins which include indoleacetic acid (IAA), naphthaleneacetic acid (NAA) and indolebutyric acid (IBA).

A shooting hormone is a class of compounds known as cytokinins which includes synthetic shooting hormones such as 6-benzylaminopurine (BAP), kinetin, zeatin, 2iP (Nβ (Δ^2-isopentenyl)-adenine *trans*-6-(4-hydroxy-3-methylbut-2-enyl) amino purine.

The inventors have determined that callus cultures are particularly suitable for regeneration because green plantlets or plants can be regenerated efficiently from callus. This is in contrast to the albino or weak, non-viable plants which have been reported in the prior art. This aspect of the present invention avoids the use of protoplasts or cell suspensions, and hence, genetically uniform cells can be used as a starting material. In addition, this avoids the problem of using physiologically fragile and nutritionally

fastidious protoplasts or cell suspensions of the prior art.

Preferably the cells are from forage grasses, more preferably from the genus *Lolium*, most preferably ryegrass.

The plant cells may be transformed by any appropriate method. Most preferably the cells are transformed by the microprojectile bombardment technique or a combination of this technique with *Agrobacterium*-mediated transformation.

Preferably, the transformed cells are callus cells produced from mature and/or developing embryos. Preferably the callus is prepared from mature and/or developing embryos obtained from seeds. More preferably the callus is grown on a callus induction medium which is based on that of Murashige and Skoog (1962) but contains 2,4-D in a range from about 1 to about 10 mg/L and more preferably about 5mg/L, L-asparagine at approximately 150 mg/L and thiamine HCl at approximately 0.5 mg/L.

Preferably, the cells chosen for transformation are the healthy cells on the callus and this is facilitated by excision of the embryo and adjacent cells. Generally, the healthy cells are selected for transformation at an age of between about 1 to about 10 weeks, more preferably about 3 to about 6 weeks such as at about 4 weeks old from cultures grown in the absence of light. However, the present invention extends to cultures earlier than 3 weeks (e.g. 1 week) or older than 6 weeks (e.g. 10 weeks).

Any suitable system may be used to identify the transformants. This may be done through a selection process by, for example, antibiotic or herbicide resistance selection or, alternatively, direct analysis of the DNA contained in putative transformants may be carried out. The methods used for direct DNA analysis will be known by those skilled in the art. Herbicide resistance selection includes but is not limited to the *Bar* gene and the Basta selection system (Hoechst), the aroA gene or EPSP gene (through overproduction of the EPSP gene) and glyphosate or the ALS gene and chlorsulfuron.

In one particular embodiment, the regeneration medium contains a shooting:rooting hormone ratio of from about 1:0 to about 10:1 and is preferably about 2:1. For example, the medium may contain from about 0.05 to 2.0 mg/L shooting hormone and from about zero to about 0.075 mg/L rooting hormone.

In another example, the medium contains about 0.5 mg/L shooting hormone and 0.025 mg/L rooting hormone. An example of suitable shooting hormone is BAP and of a rooting hormone is IAA. If only a shooting hormone is used, then BAP at approximately 0.2 mg/L to approximately 4.0 mg/L and more preferably at about 2 mg/L is appropriate.

In addition to the above, the regeneration medium preferably also contains L-asparagine at about 50 to about 750 mg/L and thiamine HCl at about 0.05 to about 5 mg/L.

The shoots may be transferred directly to soil or potting mix and grown under appropriate conditions. Preferably, the plantlets are grown at a temperature of about 15-30°C although 25°C is preferred and the cells are allowed to regenerate in light.

A particularly preferred aspect of the present invention contemplates a method of producing a transformed monocot grass comprising bombarding callus cells derived from a mature embryo of a monocot grass with a microprojectile containing a nucleic acid construct encoding a desired trait under the control of an appropriate promoter and carrying a selectable marker, under appropriate conditions to allow transformation, optionally further subjecting cells to *Agrobacterium*-mediated transfer of a similar nucleic acid construct, selecting transformed cells on a selection medium under conditions and for a time sufficient to permit regeneration of plants from the transformed cells wherein said regeneration conditions comprise shooting hormone and rooting hormone or shooting hormone alone and then culturing the resultant shoots on a medium in the absence of biologically active hormones.

The above conditions lead to regeneration of green plantlets and plants with photosynthetic ability.

The invention also extends to transgenic plants made by the methods described above.

The methods of the present invention permit for the first time, the production of monocot grasses exhibiting altered characteristics such that the modified plants have desired properties. For example, transgenic ryegrass can now be constructed having pollen with reduced allergenic properties. In this regard, antisense, cosuppression, ribozymes, regulatory genes or other suitable means may be employed to reduce expression of allergenic proteins such as *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI or isoforms thereof. The present invention extends, therefore, in a particularly preferred embodiment, to pollen from transgenic monocot grasses, such as ryegrass, exhibiting useful properties, such as exhibiting reduced allergenicity.

Accordingly, another aspect of the present invention provides a seed or seeds or pollen from a transgenic monocot grass wherein said transgenic monocot grass exhibits at least one altered characteristic when compared to a non-transgenic monocot grass of the same species.

Preferably, the transgenic monocot grass exhibits altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

Preferably, the transgenic monocot grass exhibits at least one of the following characteristics:

(a) resistance to plant pathogens;

(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;

(c) enhanced sulphur content;

(d) enhanced expression of proteinase inhibitors;

(e) expression of pigment genes;

(f) expression of regulatory genes to control indigenous genes; and

(g) reduction in levels of allergenic proteins.

Preferably, the seed or seeds or pollen which exhibits reduced allergenic properties.

Preferably, the seed or seeds or pollen which exhibits reduced levels of one or more of *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI.

Preferably, the seed or seeds or pollen is from forage grass such as ryegrass.

The invention will be further described with reference to the following non-limiting Figures and Examples.

In the figures:

Figure 1 is a photographic representation showing cell cultures and plantlets. Panel A shows embryos on callus induction medium. Panel B shows cultured callus from embryos and shoot initiation on callus. Panel C shows plantlets forming shoots; these plantlets are derived from transformed callus cells. Panel D shows a regenerated plant derived from transformed callus.

Figure 2 is a photographic representation of DNA gel blot analysis of ryegrass transformants. Each lane contains 20μg of total leaf DNA which was digested with *Eco*RI and *Bam*HI and hybridized to a ^{32}P labelled NptII gene probe. Lanes 1 and 2 contain digested DNA from non-transformed control plants. Lanes 3 to 9 contain DNA from transformed plants derived from single callus.

EXAMPLE 1

Embryo production

Embryos from *Lolium rigidum* and or *Lolium perenne* seeds were used to induce callus. Mature seed is surface sterilized in 5% v/v hypochlorite and soaked from 8 hours to 48 hours in sterile water. Mature embryos are dissected and placed on MS medium (Murashige and Skoog basal salt and minerals supplement with L-asparagine 150 mg/L, thiamine HCl 0.5 mg/L, 5mg/L 2,4D,2% w/v sucrose and 0.8% w/v agar at pH5.8) [Murashige and Skoog, 1962]. Potassium iodide may also be used at approximately 0.83 mg/L. Callus was initiated during a culture period of four weeks in dark at 25°C. Embryonic callus cultures were found to be ideal targets from microprojectile-mediated transformation because regenerable cells are not excessively shielded, and can be arranged to occupy most of the target area.

EXAMPLE 2

Regeneration of plants from competent cells after bombardment

Compact embryonic callus is transferred to MS medium containing 2% w/v sucrose, 0.5 mg/l BAP, 0.025 mg/l IAA and 0.8% w/v agar at 25°C in light. Roots were regenerated from shoots on MS medium with no hormones and containing 0.6% w/v agar. Figure 1 shows shoots regenerated from callus of ryegrass. These shoots are green.

EXAMPLE 3

An Effective microprojectile delivery system

Microprojectile bombardment conditions were optimised on the basis of frequency of transient expression in callus tissues. Parameters like microprojectile velocity, multiple bombardments and age and size of embryogenic callus were evaluated in order to achieve high efficiency of transient expressing cells. Pressure used was 24 to 29 inches Hg, distance from stopping screen was 6-13 cm and rupture disk strength was 1100 psi.

The strong moncot promoter pEmu, coupled to the GUS reporter gene (Last *et al*, 1991) was used to optimise bombardment conditions in transient assays. The GUS histochemical assay for callus tissue is performed according to Klein *et al* (1988).

For stable transformation experiments two constructs were used: the pEmu promoter couple to the npt-II (neomycin phosphotransferase) gene in order to allow selection on geneticin, kanamycin and neomycin and anther specific promoter Bgp 1 coupled to GUS in addition to the 35S promoter coupled to npt II. This plasmid also has the CaMv 35S promoter coupled to the npt-II (neomycin phosphtransferase) gene in order to allow selection on an antibiotic.

EXAMPLE 4

Selection of stably transformed tissue and regenerated plants

The concentration of the antibiotics, geneticin, and kanamycin required to inhibit the growth and to kill control untransformed callus was determined. There were found to be in the range of 15 to 50 mg/L geneticin and 15 mg/L to 50 mg/L kanamycin. Plants were regenerated from the callus lines which grown in the presence of a selection medium containing geneticin. Concentration of geneticin was the amount required to kill control (untransformed) tissue-cultured. The regenerated shoots were again exposed to medium containing geneticin and kanamycin. Surviving shoots were considered as the putative transformant shoots.

EXAMPLE 5

Confirmation of stable transformation

NPT-II ELISA assay:

The quantity of NPT-II in tissue of transformants was be measured using an NPT-II ELISA assay kit (5 prime-3 prime Inc.) Leaf tissue of transformed tissue-culture plants was used. Leaf tissue from transformed tissue-culture plants showed significant increase of NPT-II levels over untransformed control plants.

PCR analysis:

The gemonic DNA from leaf tissues of transformed plants were used from RAPD PCR analysis. Npt-II gene coding region segment was chosen from PCR amplification. Transformed plants showed the presence of amplified band of DNA which untransformed plants fail to show corresponding DNA fragment.

Southern analysis:

Integration of the introduced construct was also confirmed using southern analysis. Genomic DNA from leaf tissue was extract using a method by Dellaporta *et al* (1983).

Genomic DNA was digested with *Eco*RI and/or *Bam*HI. The digested DNA resolved and was examined for completeness of digestion by electrophoresis on a 0.7% w/v agarose gel. After transferring to a nylon membrane it was be probed with npt-II coding sequence labelled with ^{32}P. The membrane was then analysed using autoradiography. DNA from transformed plants displayed hybridisation with a single approximately 3.1-3.5 kb band while no signal was obtained from the DNA of untransformed plants (Figure 2).

EXAMPLE 6

Basta resistance selection

The construct pAHC25 which encodes the bar gene conferring phosphinothricin resistance and GUS is used to bombard 4 week old callus cells as described above. The callus cells are then selected on a suitable medium containing Basta (glufosinate ammonia) between 0.1 to 5.0 mg/L, preferably 0.5 to 5.0 mg/L and most preferably 1.0 to 3.0 mg/L. The callus cells are transferred to fresh Basta containing medium at two week intervals over a period of six weeks. Shoots are regenerated on suitable medium without herbicide or suitable medium containing 1 mg/L Basta. Regenerated shoots are then exposed to Basta to confirm tolerance. Roots are then regenerated from the shoots. Presence of the introduced DNA is then confirmed by Southern analysis.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species.

2. A transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species wherein said transgenic monocot grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic.

3. A transgenic monocot grass exhibiting at least one altered characteristic when compared to a non-transgenic monocot grass of the same species wherein said transgenic monocot grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic and wherein the callus is subjected to genetic transformation and regeneration on a solid support.

4. A transgenic monocot grass according to claim 1 or 2 or 3 exhibiting altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

5. A transgenic monocot grass according to claim 1 or 2 or 3 exhibiting at least one of the following characteristics:

(a) resistance to plant pathogens;
(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;
(c) enhanced sulphur content;
(d) enhanced expression of proteinase inhibitors;
(e) expression of pigment genes;
(f) expression of regulatory genes to control indigenous genes; and

(g) reduction in levels of allergenic proteins in, for example, pollen.

6. A transgenic monocot grass according to claim 1 or 2 or 3 wherein said grass is from a subfamily selected from *Bambosoideae, Arundinoideae, Chloridoideae, Pooideae* and *Panicoideae*.

7. A transgenic monocot grass according to claim 1 or 2 or 3 wherein said grass is from the subfamily *Pooideae*.

8. A transgenic forage grass exhibiting at least one altered characteristic when compared to a non-transgenic forage grass of the same species.

9. A transgenic forage grass exhibiting at least one altered characteristic when compared to a non-transgenic forage grass of the same species wherein said transgenic monocot grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic.

10. A transgenic forage grass exhibiting at least one altered characteristic when compared to a non-transgenic forage grass of the same species wherein said transgenic forage grass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic and wherein the callus is subjected to genetic transformation and regeneration on a solid support.

11. A transgenic monocot grass according to claim 8 or 9 or 10 exhibiting altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

12. A transgenic forage grass according to claim 8 or 9 or 10 exhibiting at least one of the following characteristics:

(a) resistance to plant pathogens;
(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;
(c) enhanced sulphur content;
(d) enhanced expression of proteinase inhibitors;
(e) expression of pigment genes;
(f) expression of regulatory genes to control indigenous genes; and
(g) reduction in levels of allergenic proteins in, for example, pollen.

13. A transgenic forage grass according to claim 8 or 9 or 10 wherein said forage grass is from *Lolium, Agaropyron, Phalaris, Poa, Festuca, Dactylis, Aleopecuris* and *Phleum.*

14. A transgenic forage grass according to claim 8 or 9 or 10 wherein said forage grass is from *Lolium.*

15. A transgenic ryegrass exhibiting at least one altered characteristic when compared to a non-transgenic ryegrass.

16. A transgenic ryegrass exhibiting at least one altered characteristic when compared to a non-transgenic ryegrass wherein said transgenic ryegrass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or *Agrobacterium*-mediated transfer of genetic material responsible for said altered characteristic.

17. A transgenic ryegrass exhibiting at least one altered characteristic when compared to a non-transgenic ryegrass wherein said transgenic ryegrass is regenerated from a callus wherein cells of said callus are subjected to microparticle bombardment and/or

Agrobacterium-mediated transfer of genetic material responsible for said altered characteristic and wherein the callus is subjected to genetic transformation and regeneration on a solid support.

18. A transgenic ryegrass according to claim 15 or 16 or 17 exhibiting altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

19. A transgenic ryegrass according to claim 15 or 16 or 17 exhibiting at least one of the following characteristics:

(a) resistance to plant pathogens;
(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;
(c) enhanced sulphur content;
(d) enhanced expression of proteinase inhibitors;
(e) expression of pigment genes;
(f) expression of regulatory genes to control indigenous genes; and
(g) reduction in levels of allergenic proteins in pollen.

20. A transgenic ryegrass according to claim 15 or 16 or 17 producing pollen exhibiting substantially reduced levels of at least one allergenic protein.

21. A transgenic ryegrass according to claim 20 wherein the allergenic protein is selected from *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI.

22. A method for producing a transgenic monocot grass, said method comprising deriving callus from a monocot grass and subjecting same to microparticle bombardment and/or *Agrobacterium*-mediated genetic transfer and then placing said callus under conditions sufficient to permit regeneration of said callus into plantlets.

23. A method for producing a transgenic monocot grass having at least one altered characteristic compared to a non-transgenic monocot grass of the same species, said method comprising obtaining callus from a monocot grass on a solid support, introducing into cells of said callus on said solid support genetic material effecting said altered characteristic *via* microparticle bombardment and/or *Agrobacterium* mediated transfer, subjecting said callus to selection conditions and then subjecting callus to regeneration conditions to form plantlets.

24. A method according to claim 22 or 23 wherein the monocot grass is from a subfamily selected from *Bambosoideae, Arundinoideae, Chloridoideae, Pooideae* and *Panicoideae*.

25. A method according to claim 22 or 23 wherein the monocot grass is from the subfamily *Pooideae*.

26. A method according to claim 22 or 23 wherein the monocot grass is ryegrass.

27. A method according to claim 22 or 23 wherein the altered characteristic exhibited is altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

28. A method according to claim 22 or 23 wherein the altered characteristic is selected from:

(a) resistance to plant pathogens;
(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;
(c) enhanced sulphur content;
(d) enhanced expression of proteinase inhibitors;
(e) expression of pigment genes;
(f) expression of regulatory genes to control indigenous genes; and

(g) reduction in levels of allergenic proteins in pollen.

29. A method of regenerating monocot grass plants from transformed plant cells, said method comprising contacting cells on a solid support upon which said cells are subjected to transformation means on a medium and under appropriate conditions and time wherein said medium contains shooting and rooting hormones and then culturing the resultant shoots on a medium in the absence of biologically active hormones.

30. A method according to claim 29 wherein the monocot grass is selected from *Lolium, Agaropyron, Phalaris, Poa, Festuca, Dactylis, Aleopecuris* and *Phleum.*

31. A method according to claim 29 wherein said monocot grass is from *Lolium.*

32. A method of producing a transformed monocot grass comprising bombarding callus cells derived from a mature embryo of a monocot grass with a microprojectile containing a nucleic acid construct encoding a desired trait under the control of an appropriate promoter and carrying a selectable marker, under appropriate conditions to allow transformation, optionally further subjecting cells to *Agrobacterium*-mediated transfer of a similar nucleic acid construct, selecting transformed cells on a selection medium under conditions and for a time sufficient to permit regeneration of plants from the transformed cells wherein said regeneration conditions comprise rooting hormone and shooting hormone and then culturing the resultant shoots on a medium in the absence of biologically active hormones.

33. A method according to claim 32 wherein the monocot grass is selected from *Lolium, Agaropyron, Phalaris, Poa, Festuca, Dactylis, Aleopecuris* and *Phleum.*

34. A method according to claim 32 wherein said monocot grass is from *Lolium.*

35. A seed or seeds or pollen from a transgenic monocot grass wherein said transgenic monocot grass exhibits at least one altered characteristic when compared to a non-transgenic monocot grass of the same species.

36. A seed or seeds or pollen according to claim 35 wherein the transgenic monocot grass exhibits altered biochemical or physiological properties, altered nutritional properties of the grass or seeds there of altered allergenicity of the grass or its seeds or pollen.

37. A seed or seeds or pollen according to claim 36 wherein the transgenic monocot grass exhibits at least one of the following characteristics:

(a) resistance to plant pathogens;
(b) improved nutritional value such as increased protein content or lower lipid content in grass seeds;
(c) enhanced sulphur content;
(d) enhanced expression of proteinase inhibitors;
(e) expression of pigment genes;
(f) expression of regulatory genes to control indigenous genes; and
(g) reduction in levels of allergenic proteins.

38. A seed or seeds or pollen according to claim 37 which exhibits reduced allergenic properties.

39. A seed or seeds or pollen according to claim 37 which exhibits reduced levels of one or more of *Lol*pI, *Lol*pII, *Lol*pIII, *Lol*pIV, *Lol*pV, *Lol*pIX or *Lol*pXI.

40. A seed or seeds or pollen according to claim 39 wherein the monocot grass is ryegrass.

FIGURE 1A

FIGURE 1B

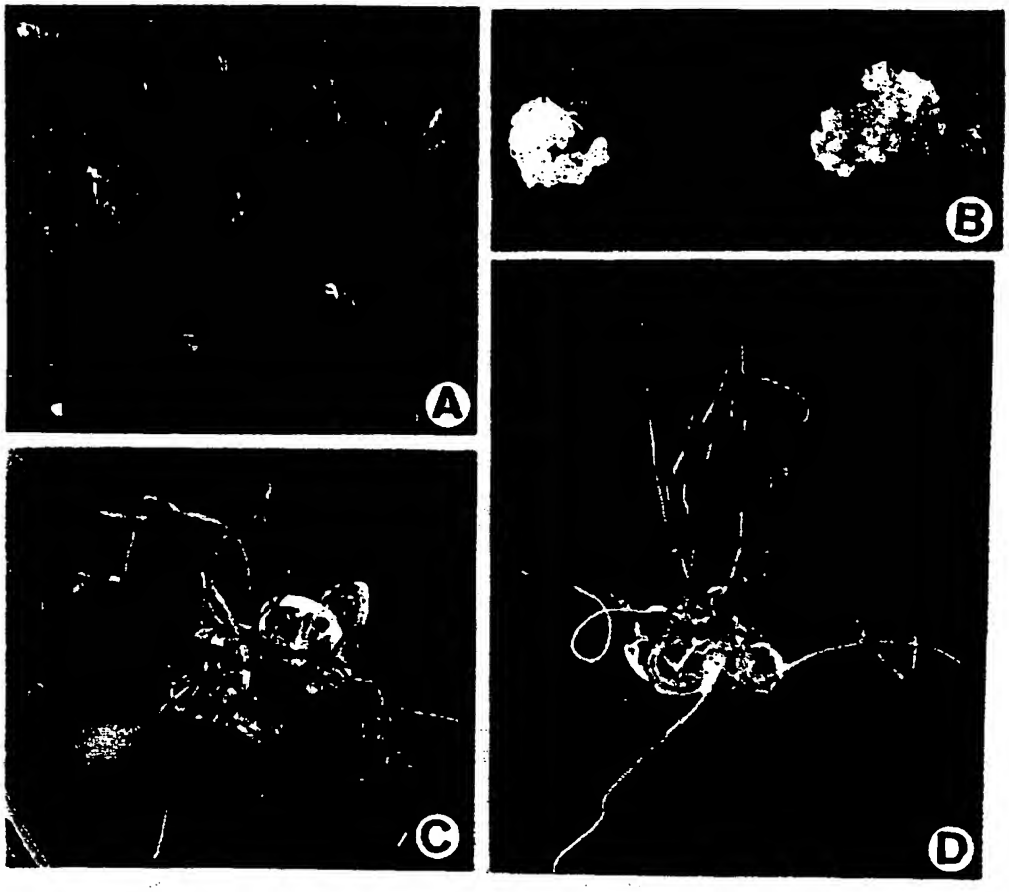
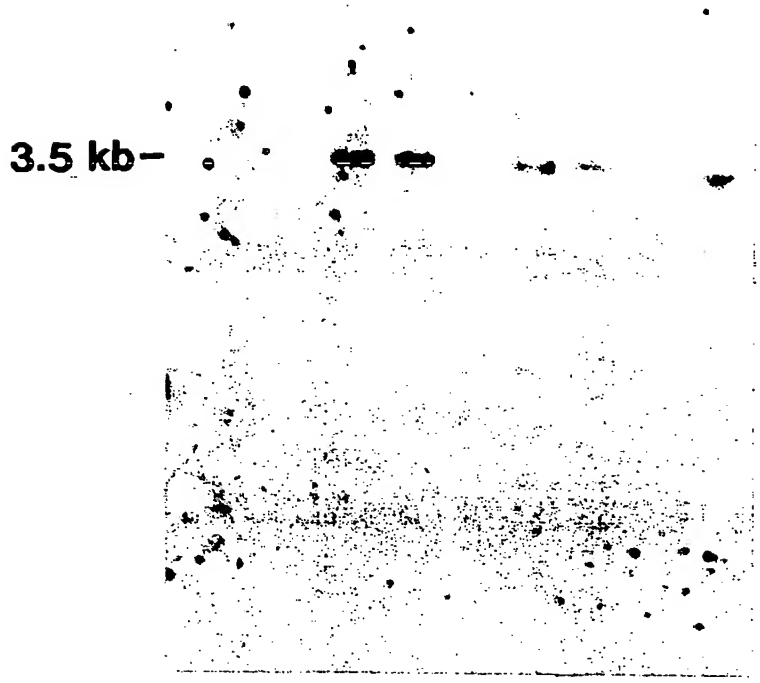


FIGURE 1C

FIGURE 1D

FIGURE 2



INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00016

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A01H 1/00; C12N 15/29

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
WPAT data base : CA database : keywords below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPM, JAPIO (Derwent) databases : keywords as below

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See attached sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Plant Science, volume 108 pages 209-217 (1995), G. Spangenberg et al "Transgenic perennial ryegrass (Lolium perenne) plants from microprojectile bombardment of embryonic suspension cells". (See whole document)	1,4,6-8,11, 13-15,18 29-31,35,36
X	Bio/Technology volume 12 (September 1994) pages 919-923, C.L. Hartman et al: "Herbicide Resistant Turfgrass (Agrostis palustris Huds.) by biolistic transformation" (see whole document)	1,4-8,11-13, 32,35-37

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
30 April 1996

Date of mailing of the international search report
29TH APRIL 1996.

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00016

Box I **Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-13, 22-25, 27-30, 32, 33, 35-38
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The scope of the claims is so broad and indeterminate that no meaningful search can be drafted for them.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00016

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Plant Cell Reports (1993) volume 13, pages 1-6. H. Zhong et al. "Transgenic plants of turfgrass (Agrostis palustris Huds.) from microprojectile bombardment of embryonic callus" (see whole document)	1-12,22-25, 27-29,32,35, 36,37
X	Bio/Technology volume 10 (June 1992) pages 667-674. V. Vasil et al: "Herbicide Resistant Fertile Transgenic Wheat plants obtained by microprojectile bombardment of regenerable embryonic callus" (see whole document).	1-4,6,7,12, 22-25,27-29, 32,35,36
Y	Genetic Engineering of plants for Crop Improvement (1993) by Rup Lal and Sukanya Lal, CRC Press Inc, USA Chapter 3, "Engineering herbicide Resistance into Plants", chapter 4 "Engineered resistance against plant virus disease" and chapter 5 "Improvement of the nutritional quality of plants by manipulation of seed storage genes". (pp85-217)	4,5,12,19 28,36,37
Y	Plant Molecular Biology volume 24, pages 401-405 (1994). H.M. van der Maas et al: "Stable Transformation and long-term expression of the gus A reporter gene in callus lines of perennial ryegrass (Lolium perenne L.)" (see whole document).	1-11,12-19, 22-37
Y	J. Plant Physiol. Volume 140, pages 101-105 (1992). O.M. Faiz Zaghmout and W.A. Torello: "Plant Regeneration from callus and protoplasts of perennial ryegrass (Lolium Perenne L.) (see whole document).	1-11,12-19, 22-37
Y	Res. Bull. Obihiro Univ. Volume 18, pages 143-146. Y. Horikawa et al: "Expression of Gus-gene in monocot temperate grasses by an improved particle gun" (see whole document, in particular column 2, 3rd paragraph, page 32).	1-11,12-19, 22-37
A	WO,A,93/04174 (The University of Melbourne) 4 March 1993 (see whole document)	4,5,12,18-21, 27,28, 36-39

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 96/00016

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	93/04174	AU	24409/92	EP	665888	JP	6509941

END OF ANNEX

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00016

Box B. FIELDS SEARCHED

WPAT : USPM : JAPIO : keywords

Search 1:

SS1 monocot: or grass: or maize# or rice# or wheat# or Lolium or Festuca or Dactylis or bamboo: or ryegrass: or rye or oat# or barley or corn: or Fescue or Millet or Agaropyran or phalaris or poa or aleopucuris or phleum or sorghum or reed# or sugar (w) cane#.AND

SS2 transgen: or gene: or DNA or RNA or nucleic (W) acid# or mutat: or transform: or modif: AND

SS3 A01H /IC or A01G 7/IC or A01G 9/IC AND

SS5 CALLI or CALLUS

Search 2:

Lolium or ryegrass and SS2 above.

Search 3:

monocot: and grass: and SS2 above.

CAS On-line

keywords as above (search 1) and 1991-1996.